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Intensifying chitin hydrolysis by adjunct treatments – an overview

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Abstract

Chitin is, after cellulose, the most abundant organic natural polysaccharide on Earth, being synthesized as a dominant component in the exoskeletons of crustaceans, among other sources. In the processing of seafood for human consumption, between 40 and 50% of the total raw material mass is wasted, causing a significant problem for the environment due to its slow degradation. Efforts to find uses for chitin derivatives, particularly their oligomers, have intensified since these chemicals are highly functional and offer a wide range of applications, especially as antimicrobial agent. As a consequence, some adjunct treatments, either chemical or physical in

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nature, have been employed to assist acid and enzymatic hydrolysis. This work provides a detailed review of the methods employed to intensify the formation of chitin oligomers, particularly focusing on the adjunct treatments used (microwave, ultrasonication, steam explosion and gamma irradiation), and evaluate the yield and characteristics of the oligomers formed. Adjunct treatments are more suitable for enzymatic hydrolysis since these treatments modify the chitin structure, and enhance the hydrolysis rate and yield of the oligomers, under milder reaction conditions. For future research, it would be worth trying pre-treatments like the application of high-pressure to chitin in order to lower its crystallinity.

Keywords: Chitin hydrolysis; Oligomers; acid hydrolysis; enzymatic hydrolysis; pretreatment

INTRODUCTION

Chitin - a β-(1→4)-linked polymer composed predominantly of *N*-acetyl-D-glucosamine (GlcNAc) units – is, after cellulose, the most abundant organic natural polysaccharide on Earth, offering a broad range of structural and protection functions, like cellulose in plants. The structure of chitin differs from that of cellulose in that the C-2 hydroxyl residues (-OH) are replaced by acetamide groups (CH_3CONH-) (Figure 1). Chitin comprises three polymorphs: α-chitin is the most abundant and β- and γ-chitin are very rare, which can be distinguished by their molecular chain arrangement and hydrogen bonding systems. α- and β-Chitins have an antiparallel and a parallel chain arrangement, respectively, whereas γ-chitin consists of both parallel and antiparallel chains.[1,2] The antiparallel chain in α-chitin are arranged in bonded piles or sheets linked together by the hydrogen and

acetamide groups running in opposite directions as compared to the β-chitin.[2] The intermolecular and hydrogen bonds present in α-chitin make it difficult to melt and dissolve in common solvents at normal temperatures, which makes this material inconvenient for further processing.[1]

Chitin is synthesized as a dominant component in the exoskeletons of crustaceans and insects, as well as in the cell wall of fungi, yeast and algae. Nowadays, chitin extracted from crustacean shells, such as crab, shrimp, prawn, krill and lobster, are readily available in large quantities from shellfish processing industries in comparison with other sources. In the processing of seafood for human consumption, between 40 and 50 % of the total raw material mass is wasted.[3] This quantity of waste has been reported to be between 10^{10} and 10^{11} tons per year, which poses a significant problem for the environment due to its slow degradation.[3-6]

Crustacean shells are composed of proteins, chitin, minerals, and carotenoids, which are the major components based on the dry mass.[3] In order to extract chitin from the crustacean shells, the following main steps must be employed: demineralization, deproteinization and decolouration.[7] The demineralization of shells can be achieved by extraction with dilute acid (hydrochloric acid, formic acid, acetic acid, sulfuric acid or EDTA – ethylenediamine-tetra-acetic acid) at room temperature. Deproteinization can be effected by treating the demineralized waste with aqueous alkali solution (sodium or potassium hydroxide) at the temperatures between 65 and 100^{o}C. In this step, the most significant parameters to be considered for an efficient deproteinization are the concentration of alkali solution, processing time and temperature, and solid to solvent ratio. Benhabiles et *al*.[8] reported that the conditions for 96 % protein removal were: processing time of 120 mins at a temperature of 45

˚C and the use of solid to solvent ratio of 1:2 (w/v) with the solvent (NaOH solution) concentration being 2 M. Finally, the decolouration was carried out by a bleaching with activated charcoal, or by using strong oxidizing agents such as sodium hypochlorite (NaClO) or hydrogen peroxide (H_2O_2) solutions.[9] Chitin separated from minerals, colourants and proteins is commercially available for industrial uses, although, it may require further purification in order to obtain regenerated chitin, which can be effectively used for producing chitosan and oligomers.[10-12]

Chitosan - a β-(1→4)-linked polymer composed predominantly of D-glucosamine (GlcN) units - is an *N*-deacetylated derivative of chitin formed by deacetylation under alkaline conditions at elevated temperature, which sodium hydroxide is a common alkaline media.[4,13-15] Chitosan is a semi-crystalline polymer which is insoluble in aqueous solutions above a pH value of 6.5, but fully soluble in diluted acids below pH of 5.[16,17] The difference between chitin and chitosan can be defined in terms of the ratio of 2-acetamido-2-deoxy-D-glucopyranose to 2-amino-2-deoxy-D-glucopyranose units, which is commonly known as degree of *N*-acetylation (DA). The DA of chitosan is typically less than 0.35, whereas that of chitin is normally above 0.90.[15] Higher and lower molecular weight chitosans may also possess excellent properties for specific applications. Numerous studies have reported that chitosan with higher molecular weight possess superior mechanical properties, such as higher tensile strength and better elongation of chitosan film,[18,19] higher antifungal activity,[20,21] and enhances nasal absorption of peptide drugs[22]. Lower molecular weight chitosans have a stronger superoxide scavenging activity,[23] greater antimicrobial activity,[24] and higher permeability of film.[18]

Chitin oligomers are derived from chitin by depolymerization in the presence of acid or enzymes.[4,13,14] Chitin oligomers are composed of GlcNAc units with approximately ten residues or less, and can be produced by depolymerization of chitin.[4] The depolymerization is commonly achieved by acid and enzymatic hydrolysis, employing hydrochloric acid (HCl) and chitinases, respectively. The chitin polymer chain is cleaved by this reaction to become oligomeric. Both these hydrolysis methods have been extensively reviewed by Jeon et *al*., Prashanth and Tharanathan and Ahmed et *al*.[4,25,26] Efforts to find uses for chitin oligomers have intensified since these chemicals are highly functional and offer a wide range of applications. Chitin oligomers have received increased research and commercial attention because these molecules are not only water soluble, nontoxic and biocompatible, but also exhibit numerous biological properties, such as antibacterial, antifungal, antitumor, and antioxidant activities.[27,28-30] According to the literature, antimicrobial action is one of the most important property of chitin oligomers due to its water solubility, and potent activity against bacteria and moulds. It has been reported that the oligomer possess low minimal inhibitory concentrations (MIC) values for gram-positive strain (*S.aureus, B. subtilis, L. monocytogenes and B. cereus*) and gram-negative strains (*E. coli, V. cholera, Shigella disenteriae, B. fragilis, P. aeruginosa,* and *P. melaninogenica*) compared to chitin and chitosan.[27,31] In another research, the chitin oligomers were found to possess antifungal activity against *Aspergillus niger*.[28] Besides antimicrobial activity, some works also reported that the chitin oligomers, with molecular weight of 1-5 kDa, possess the ability to inhibit membrane protein oxidation and act as potent antioxidant in live cells.[30,32]

As a consequence of growing interest for converting chitin into its oligomers, some adjunct treatments, either chemical or physical in nature, have been employed to assist acid and enzymatic hydrolysis, as well as enhance the yield of oligomers. Gamma irradiation, ultrasonication, microwave irradiation, steam explosion, supercritical water, grinding and depressurization are physical methods that have been employed previously to facilitate chitin hydrolysis.[33-38] Aqueous solutions such as phosphoric acid, hydrochloric acid, alkaline solution and methanol modify chitin structures prior to hydrolysis. These chemical treatments have been reported to decrystallise chitin, increase its solubility, and accelerate subsequent enzyme hydrolysis.[11,12,39]

This article aims to review the methods which have been employed to intensify the formation of chitin oligomers, particularly focusing on the adjunct treatments to improve the hydrolysis, and evaluate the characteristics of the chitin and oligomers formed.

PREPARATION OF CHITIN OLIGOMERS

This section reviews the different hydrolysis methods employed and adjunct treatments proposed to assist depolymerization of chitin.

Methods based on acidic hydrolysis

Chitin oligomers are generally manufactured by acid hydrolysis of chitin employing strong acids such as concentrated hydrochloric acid (HCl) to effect the cleavage of the chitin polymeric chain.[27,30,34,40-42] The concentration of HCl, incubation time and temperature are the key parameters in the process. HCl concentrations ranging from 3 to 12 N have been used to hydrolyze chitin at temperatures ranging between 20

and 90 ºC for time durations ranging between 5 min and 7 h.[27,30,32,40-42,44,45] The processing steps employed by earlier researchers for hydrolysing chitin are summarized in Figure 2. In general, a given amount of chitin powder is added to HCl solution and the mixture is constantly stirred under reflux in a water bath maintained at the desired temperature.[32,40,42] After incubating thus for the desired time period, the hydrolysis is stopped by cooling the reaction mixture in an ice bath or on dry ice,[40,44] following which, the chitin oligomers are isolated by: 1) freeze-drying the solution under vacuum, 2) redissolving the dried product in deionised water, and 3) neutralising the solution with NaOH followed by filtration to remove impurities from the oligomers. The freeze-drying and redissolving steps have been repeated twice by some researchers to remove any residual HCl left.[42] Some researchers have also proposed neutralisation with 25% sodium hydroxide (NaOH) to stop the hydrolysis.[30,32] The neutralized solution is centrifuged to separate the supernatant, and the unhydrolyzed chitin and the supernatant are desalted and purified prior to spray-drying in order to obtain the chitin oligomers as a light yellow powder.

Recently, Kazami et *al*.[45] developed an acetone precipitation method as a replacement to NaOH neutralisation to claim a simpler procedure for isolating the chitin oligomers. Acetone-insoluble material can be recovered by the following steps: 1) adding acetone to stop the hydrolysis and stirring at a low temperature for a day; 2) centrifuging the mixture of acetone and chitin; 3) repeatedly washing the acetone-precipitate with acetone to remove HCl (until the pH of supernatant reaches 4-5); and 4) suspending the acetone-precipitate in cold diethyl ether, centrifuging and drying to constant weight.

The dried acetone-precipitate (acetone-insoluble material) is then mixed with water in order to extract the water-soluble chitin oligomers as follows: 1) mixing the dried acetone-precipitate with water, stirring overnight, and centrifuging in order to separate the supernatant and water-precipitate; 2) repeating step (1) for the water-precipitate; 3) combining supernatant recovered from steps (1) and (2) as water-soluble chitin oligomers; and 4) suspending the water-precipitate in cold diethyl ether, centrifuging and drying to constant weight. Generally, the supernatant containing the chitin oligomers obtained appear brown or yellow in colour. Therefore, activated charcoal treatment is applied to yield a clear solution prior to the final drying process.[34,42]

Chitosan can also be potentially converted into chitin oligomers by employing a two-step process involving: 1) depolymerization of chitosan by hydrolysis in HCl to form chitosan oligomers; and 2) partial *N*-acetylation of chitosan oligomer in hydro-alcoholic solution of acetic anhydride in order to produce the oligomers.[42] This approach generally requires numerous steps and produces HCl and acetic anhydride residues, which are undesirable products and have a significant impact on the environment.

Figure 3 shows a possible mechanism of chitin hydrolysis by concentrated HCl that has been reported previously by Kazami et *al*.[45] The chitin before hydrolysis is assumed to consist of alternating crystalline and amorphous regions, and composed a number of polymeric chains. Initially, the amorphous regions are rapidly cleaved within 5 min of hydrolysis to produce regular-sized segments with a central crystalline region attached to amorphous tails at both ends. The amorphous tails are then gradually degraded, leading to the accumulation of chitin oligomers, as well as

a crystalline chitin core consisting of multiple chitin chains. Single chitin chains may then be slowly separated from the chitin core, and once separated, be rapidly hydrolyzed to yield chitin oligomers within 30 to 60 min.

Although the hydrolysis process described above is effective, some disadvantages have been reported such as the occurrence of deacetylation (that produces chitosan oligomers instead of chitin oligomers), production of acidic waste streams, high cost, lower yield of high degree of polymerization (DP) oligomers, and requiring skilled labor force for purification.[4,11,25] The lower yield and shorter chain length of the oligomers formed, particularly dimers, adversely influence bioactivity.[46] In addition, the process costs and the environmental impact of the process are also high mainly due to the use of strong acids during hydrolysis.[6]

Methods based on enzymatic hydrolysis

In contrast to acidic hydrolysis, higher DP chitin oligomers can be produced under milder reaction conditions by employing enzymatic hydrolysis. Enzymes, mainly chitinases, which have higher chitinolytic activity, are used for this purpose, and are commonly produced from microorganisms, plants, and insects. Some researchers have used bacterium *Serratia proteamaculans* 568,[47] *Serratia marcescens* 2170,[48] *Rhizobium* sp. GRH2,[49] *Bacillus cereus* TKU027,[6] to produce chitinases, while others have used hevamine,[50] a plant enzyme, having both chitinase and lysozyme activities. Chitinases can also be successfully extracted from fungi such as *Lecanicillium lecanii* and *Lecanicillium fungicola*, while *Trichoderma reesei* fungi is reported to have hydrolases (cellulases and β-glucanases).[11,36,39] The enzymes produced from various sources have to be purified before being used for hydrolysis.

Additionally, non-chitinase commercially available enzymes, like cellulase, hemicellulase, pepsin, papain, lysozyme, and pectinase have also been reported to hydrolyse chitin.[12,31,51]

A flow diagram for the enzymatic hydrolysis of chitin is showed in Figure 4. Prior to hydrolysis, the substrate or chitin suspension is prepared by adding chitin powder to a phosphate or acetate buffer solution, so that its concentration is between 0.5 and 2.0 % w/v.[6,12,33,48,51] Buffers strength in the range of 0.01 to 0.05 M and pH 5.0 to 5.5, are reported to provide optimum condition for the substrate preparation.[6,12,31,36,51] The enzyme is subsequently mixed with the substrate at an appropriate amount, so that its chitinolytic activity in the system is under the standard assay condition. In the hydrolysis, one unit of enzyme activity (1 U) is defined as the amount of enzyme releasing 1 μmol GlcNAc per minute.[52] After mixing, the mixture is incubated at various temperature-time combinations, depending on the enzyme action. Hydrolysis by chitinases, lysozyme, pectinase, and pepsin have been reported to require incubation at temperatures between 37 and 44 ºC.[6,12,36,47,48,50,51] To stop the reaction, the hydrolysis mixture is heated to 90 ºC or boiled for 10 min, and subsequently centrifuged and filtered to separate the supernatant which contains the oligomers, and unhydrolysed chitin.[11,31,36,51]

Earlier studies have reported that the yield of high DP chitin oligomer resulting from enzymatic hydrolysis is greater compared to acidic hydrolysis.[4] This may be due to the enzyme acting selectively on the crystalline and otherwise inaccessible parts of chitin. During chitin hydrolysis, the enzymes which degrade the polysaccharide chain can be either endo-acting or exo-acting. Figure 5 shows the mechanism of endo-acting enzymes which randomly cleave glycosidic linkages of chitin, generate free

ends and chitin oligomers, while exo-acting enzymes release dimers (two units of GlcNAc) from the reducing (C1) or non-reducing (C4) ends.[47]

Of course, the use of enzymes also has its fair share of disadvantages: specific enzymes such as chitinase and chitosanases are not readily available commercially and, even if available, tend to be very expensive.[37] Further, the presence of protein residues after hydrolysis potentially limit biomedical application due to possible allergen and pyrogenicity[25] effects, which will warrant significant further purification that will make the whole process economically unviable. However, this method has a key advantage because minimum chemical wastes are produced during hydrolysis.[6]

Use of chemical and physical adjunct treatments to intensify chitin hydrolysis

Irradiation of chitin has recently been used to assist acid or enzymatic hydrolysis. Ultrasonic irradiation or ultrasonication has been reported to be advantageous for depolymerization, because it preserves the chemical nature of the polysaccharide by simply splitting the most susceptible chemical bonds and lowering its molecular weight.[53] Takahashi et *al*.[54] and Ajavakom et *al*.[34] determined the effectiveness of ultrasonication during acid hydrolysis. In these investigations, the mixture of chitin and HCl was sonicated at various wavelengths for different durations. It has been found that the chitin powder completely dissolved within 30 min in the HCl solution during sonication at 50 or 60 Hz (275 W).[34] Takahashi et *al*.[54] noted that the amount of oligomers up to DP 7 (seven units of GlcNAc in each chain) increased after 120 min of ultrasonication, which was 2 to 4 times greater than oligomers produced without ultrasonication. On the other hand, the chitin could be degraded during demineralization in an ultrasound-assisted extraction process. The depolymerization

occurring may be due to the application of high intensity irradiation, which results in breaking covalent bonds in the polymeric chain.[55] Ultrasonication has also been applied to the chitin suspension prior to enzymatic hydrolysis. In this process, the treated chitin depolymerized to a lesser extent, which was detected by the lower amount of reducing sugars measured.[36] All these studies agreed that ultrasonication facilitated acidic and enzymatic hydrolysis without drastically changing the degree of acetylation (DA) of the chitin. However, if high intensity ultrasonication was applied, the covalent bond in the polymeric chains of chitin could break due to cavitation by temporarily dispersing aggregates.[55]

Some researchers have measured ultrasonic intensity directly, while others have not. When the intensity is not measured, it is calculated by measuring the transient rise in temperatures during ultrasonication, and after it is switched off, as follows:

$$I = \frac{mc_p}{\pi r^2}\left[\left(\frac{\mathrm{d}T}{\mathrm{d}t}\right)_a - \left(\frac{\mathrm{d}T}{\mathrm{d}t}\right)_b\right] \quad (1)$$

where: I is the ultrasonic intensity, $(\mathrm{d}T/\mathrm{d}t)_a$ is the slope of the initial rise in temperature, $(\mathrm{d}T/\mathrm{d}t)_b$ is the slope of heat loss after the ultrasonic processor was turned off, m is the sample mass, c_p is the heat capacity of the solvent, and r is the radius of ultrasonic probe.[55]

Microwave irradiation has been established as a patented technique for producing chitin oligomers. Chitin is added to HCl and subjected to a conventional microwave device at 700 to 2100 W for up to 24 h.[56] This technique has been repeated by other researchers with a slight modification, where 38% HCl was initially pre-warmed at 850 W by conventional microwave oven for a shorter time and the pre-warmed HCl

was quickly added to chitin powder for further irradiation at various reaction times.[34] Microwave irradiation has also been applied prior to the enzymatic hydrolysis of chitin. Roy et *al.*[33] used a microwave with built-in magnetic stirrer and non-contact infrared continuous feedback temperature system for chitin pre-treatment. In this study, the chitin suspended in acetate or phosphate buffer was irradiated with the microwave at optimum temperature and time reported to be 57.5 ºC and 38 min, respectively; this was followed by hydrolysis with chitinase. This study found that the polar molecules in the chitin suspension align with the magnetic field generated by microwave, and have a tendency to accelerate the hydrolytic reaction rate. The microwave pre-treatment is comparable to ultrasonication, when the treated chitin is insignificantly deacetylated after irradiation.[33]

Improvement on the hydrolysis rate and chitin properties could be accomplished by gamma irradiation, which is one of the physical methods requiring no chemical additive and no temperature control during reaction.[37,38] Gamma irradiation, applied at different doses ranging from 15 to 210 kGy, to the solid form of chitin, prior to hydrolysis with chitinases, has been investigated.[37] Previously, this method was effectively applied as the adjunct treatment in the production of chitosan oligomer, using irradiation doses ranging between 2 and 500 kGy.[57-59] These authors suggested that the application of gamma irradiation at various doses may reduce the molecular weight of oligomers due to the breaking of glycoside bond.

The adjunct treatments discussed above are physical methods which influence the reaction through non-thermal effects. However, thermal treatments, such as steam explosion (SE), has also been investigated to influence the enzymatic hydrolysis of chitin. Steam explosion treatment consists in heating of chitin with saturated steam,

followed by a sudden decompression of the pressurized system to produce insoluble solid fraction and a liquid fraction of soluble sugars.[60,61] Villa-Lerma et *al*.[36] applied steam explosion to the mixture of chitin powder and deionized water at 180 ºC and 1 MPa for various reaction times. The treated chitin mixture was then added to phosphate buffer and this combination acted as substrate for hydrolysis with chitinase. It has been reported that steam explosion can significantly reduce chitin crystallinity without significant depolymerization occurring during treatment.

Another physical treatment that has been proposed to enhance and increase the oligomers yield is a combination of the use of supercritical water and mechano-chemical grinding with a ball mill.[35] Supercritical water is water at temperatures near or above 374 ˚C, meanwhile, the mechano-chemical grinding is a term used for the chemical reaction that occurs during mechanical treatment of the sample, typically grinding by ball mill.[62-64] After being treated with supercritical water, the chitin undergoes mechano-chemical grinding. This pre-treatment results in chitin flakes which are fragile and easy to grind, and form an effective substrate for hydrolysis with reduced particle size and molecular weight.[35]

In addition, chitin structures can also be pre-treated chemically by using aqueous solution, such as phosphoric acid, HCl, alkaline solution (mixture of sodium hydroxide and sodium dodecylsulfate), and methanol. These pre-treatments have reportedly decrystallized chitin, increased its solubility, and accelerated subsequent enzyme hydrolysis.[11,12,39] Ramírez-Coutiño et *al*.[39] deacetylated α and β-chitin by deacetylation with alkali solution, thereby partially transforming chitin into chitosan (degree of deacetylation 55 and 50%, respectively) prior to hydrolysis. Reduction in

hydrogen bonds caused by the elimination of acetyl group increases the solubility of partially deacetylated chitin in aqueous media.[65,66]

CHARACTERISTICS AND CHARACTERIZATION OF CHITIN AND ITS OLIGOMERS

This section summarizes the various methods used in literature to characterize chitin oligomers produced by chitin hydrolysis with and without the use of adjunct processes. Chemical structure and composition, degree of N-acetylation (DA), degree of polymerization (DP), molecular weight, and crystallinity are important properties characterizing chitin oligomers. The methods employed to determine these characteristics are discussed below.

Chemical structure and composition

Fourier transform infrared (FT-IR) and proton nuclear magnetic resonance H NMR spectroscopies can be reliably used to record the composition and chemical structure of the oligomers.[30,32,34,36,37,39,41-43,55] These methods require simpler procedures to prepare samples, and provide information on the chemical structure faster than conventional methods.[67]

Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectroscopy is a very attractive technique suitable for both soluble and insoluble samples.[68] The technique is based on the vibrations of the atoms of a sample molecule. Infrared spectrum is obtained by passing infrared radiation through the sample and determining the fraction of the incident radiation absorbed at a

particular energy. The energy at any peak in an absorption spectrum corresponds to the frequency of a vibration of a part of the sample.[69]

The infrared spectrum can be divided into three main frequencies which is far-infrared (< 400 cm^{-1}), mid-infrared (4000 - 400 cm^{-1}) and near-infrared (13000 – 4000 cm^{-1}). To obtain the FT-IR spectrum of the chitin and its oligomers, mid-infrared frequency is used as explained by Lambert-Beer Law.[27,30,36,55,70] The law states that the absorbance values at a given frequency of 4000 to 400 cm^{-1} are proportional to the concentrations of the sample. Measurements are initiated by preparing KBr (potassium bromide) pellet, which is a small amount of chitin sample well mixed with the KBr (approximately 0.1 to 1.0 % w/w) and pressed into a pellet-forming die. The measurement is taken by scattering the infrared light onto the KBr pellet using FT-IR system.[30,70,71] This is the most common method used, which utilizes the property of KBr as alkali halide that becomes plastic when subjected to pressure and forms a transparent sheet in the infrared region.[72]

In a chitin molecule, the acetamido group at the position C2 (Figure 5), intramolecular hydrogen bonds (C6-OH···O=C) and intermolecular hydrogen bonds (NH···C=O) provide polymer stability.[73] In α form, the absorption bands of carbonyl group (amide I) stretching are split at 1660 cm^{-1} and 1620 cm^{-1}, which are attributed to intermolecular and intramolecular hydrogen bonds respectively.[73,74] Unlike α crystalline form, a single absorption band of amide I β-chitin is observed at 1560 cm^{-1} which is attributed to the stretching of carbonyl group hydrogen bonded to amide group of the intra-sheet chain.[74] In previous research, α-chitin extracted from *Daphnia magna* resting egg (zooplankton genus in aquatic ecosystems) exhibited absorption bands at 1652 cm^{-1} and 1622 cm^{-1} for amide I.[73] Recently, the chitin

extracted from six different aquatic invertebrates presented the spectrum of amide I at 1652-1656 cm^{-1} and 1619-1620 cm^{-1}, thus allowing them to be characterized as α crystalline form.[75] These two strong absorption bands were also observed in ultrasonication and steam explosion treated chitin. However, an additional shoulder was present at 1633 cm^{-1} in ultrasonication treated chitin which might be due to a reduction in amino-based hydrogen bonding.[36] In chitin oligomers structure, the spectrum generated by FT-IR exhibits intense absorption bands at 3358 cm^{-1} (OH stretch), 2917 cm^{-1} (C-H stretch), 1651 cm^{-1} (C=O stretch, amide I), 1548 cm^{-1} (N-H bend, amide II), 1313 cm^{-1} (C-N stretch, amide III), and 1150-1000 cm^{-1} (pyranose), as shown in Figure 6.[30] All the reported FT-IR spectra indicate that the crystalline structure formation of intact chitin, treated chitin and its oligomers are not affected by the types of processes.

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy

^{1}H NMR spectroscopy is a powerful and reliable technique for polymer structural analysis by exploiting the magnetic properties of certain atomic nuclei. The spectra of chitin and its oligomers can be obtained by cross-polarization or magic-angle-spinning method using deuterated oxide (D_2O) as solvent.[31]

In chitin, the chemical composition obtained by ^{1}H NMR resonated between 3.6 and 4.4 ppm, which are assigned to H-2 to H-6 protons, while acetyl protons are found at 2.6 ppm.[74,43] The anomeric region of α- and β-anomer (H-1) generated peaks at 5.43 and 5.05 ppm, respectively.[43] As observed in chitin oligomers, the spectra present two singlets at 2.06/2.08 ppm assigned to the N-acetyl protons, and a broad signal at 5.20 ppm assigned to H-1 protons of the reducing end α anomer residues.[42] The

chitin oligomers produced by enzymatic hydrolysis of chitin using commercial enzymes possessed obvious resonance peak at 1.9 ppm assigned to the acetyl group, 3.4 ppm detected for H-2 and 3.5 to 3.8 ppm corresponding to H-3, H-4, H-5 and H-6 protons, meanwhile the H-1 (β) and H-1 (α) protons generated peaks at around 4.6 to 4.7 ppm and 5.2 ppm, respectively.[31] Ngo et *al*.[30] reported the resonance of chitin oligomers hydrolyzed by acid (HCl) at 1.97 ppm is assigned to acetyl group, 3.34 ppm is corresponded to H-2 , 3.37 to 3.77 ppm are detected for H-3, 4, 5, 6, 4.5 to 4.6 ppm is assigned to H-1 (β) and 5.0 ppm is corresponded to H-1 (α). All spectra of chitin are essentially identical to the oligomers, regardless of the type of hydrolysis. It is caused by the occurrence of glyosidic linkages breakdown during the hydrolysis without the interference of deacetylation.[41]

Degree of *N*-acetylation

The degree of *N*-acetylation (DA) represents the molar fraction of *N*-acetylated units in the chitin polymer chain.[76] The DA is an important parameter influencing physico-chemical, electrostatic and biological properties of chitin.[77] Generally, the DA can be determined by the calculation of the absorbance and intensities generated by FT-IR and NMR, respectively. The absorbance values (A) obtained by FT-IR can be used to measure DA by using the following equation:

$$\% \, N\text{-acetylation} = (A_{1655}/A_{3450})(100/1.33) \quad (2)$$

where, A_{1655} and A_{3450} are the absorbances at 1655 cm^{-1} of the amide-I band and 3450 cm^{-1} of the hydroxyl band, respectively; and the factor of 1.33 is the ratio of A_{1655}/A_{3450} for fully *N*-acetylated chitin.[55] However, Chang et *al*.[40] determined the

percentage of DA by using the following equation, originally proposed by Baxter et *al*.[78]:

$$\% \textit{N}\text{-acetylation} = (A_{1655}/A_{3450})(115) \quad (3)$$

The values of 100/1.33 (i.e. 75.2) and 115 are reciprocal values of the slope of the linear section of the plot of absorption ratio (A_{1655}/A_{3450}) against DA, which depend on the baselines used.[67,70] Eq (3) is more reliable in comparison with Eq (2), since Eq (2) tended to overestimate values for DA > 20.[67]

The DA can also be quantitatively analysed by carbon nuclear resonance magnetic (C-NMR), where the relative intensities determined for the resonance of the ring carbon (I_{C1}, I_{C2}, I_{C3}, I_{C4}, I_{C5}, I_{C6}) and methyl carbon (I_{CH3}) is used in the following equation proposed by Ottey et *al*.[79]:

$$\% \text{ N-acetylation} = \frac{I_{\mathrm{CH3}}}{(I_{\mathrm{C1}}+I_{\mathrm{C2}}+I_{\mathrm{C3}}+I_{\mathrm{C4}}+I_{\mathrm{C5}}+I_{\mathrm{C6}})/6} \times 100 \quad (4)$$

Eqn (4) has been applied in earlier work on chitin.[68,70,80]

Fully acetylated chitin provides DA of 100% while 0% corresponds to completely deacetylated chitin (chitosan).[67] In previous work, the DA of chitin decreased from 98% to 93, 88 and 73% after exposure to ultrasonication, steam explosion and depressurization, respectively.[36,38] The lower DA values after adjunct treatment indicate deacetylation and modification of chitin structure. However, the lowest DA of depressurized chitin gave higher yield of the oligomers. It has been reported that the DA values of chitin between 45 and 55% provide excellent solubility in aqueous media.[38] Some authors have reported that the DA between 40 and 60% possessed

suitable characteristics for enzymatic hydrolysis, where the chitin solubility reached was greater than 60%.[39,81] It caused the polarity and electrostatic repulsion of the amino groups increased, thus increasing the accessibility of chitin to enzymatic attack.[39]

Degree of polymerization

Degree of polymerization (DP) is a significant parameter to identify the number of monomeric units in the oligomers. MALDI-TOF mass spectrometry is a powerful technique, which has been extensively applied to determine the DP of chitin and chitosan oligomers.[6,36,42,82,83] Figure 7 shows the MALDI-TOF spectra which consists of high intensity of a number of peaks assigned to a certain DP. The DP of the oligomers can be determined by the peak-to-peak mass difference of 203, which is the GlcNAc repeating unit ($C_8H_{13}NO_5$). The end-groups of H and OH are deduced from monoisotopic mass. Each DP can be calculated from a peak with particular mass unit (m/z) as follows:

$$DP = (\text{mass unit of a peak} - H - OH - Na) / C_8H_{13}NO_5 \quad (5)$$

where DP is the degree of polymerization. The molecular mass of $C_8H_{13}NO_5$ is 203 (i.e. mass unit of chitin oligomers); the atomic mass of H is 1 (hydrogen); and those of the OH group and Na are 17 and 22.99, respectively.[42]

The DP can also be determined by using high performance liquid chromatography (HPLC), where the concentration of each DP present can be calculated from the peak areas in the HPLC profile using the standard curve obtained from pure chitin monomer, dimer, trimer, tetramer, pentamer, and hexamer standard solution.[6,11,12,40,57] Moreover, the DP can be quantitatively analysed by integrating the

signals of the anomeric protons (H-1 (α) and H-1 (β)) obtained by ^{1}H NMR using the following equation:

$$DP_n = [\int H\text{-}1\ (\alpha) + \int H\text{-}1\ (\beta) + \int H\text{-}1c] / [\int H\text{-}1\ (\alpha) + \int H\text{-}1\ (\beta)] \quad (6)$$

where ∫H-1 (α) and ∫H-1 (β) refer to the integral of the H1 protons of the chitin oligomers at terminal reducing end having α and β configuration, respectively, and ∫H-1c is the sum of integrals of the H1 protons of all the central units.[84]

As reported by Chang et *al*.[40], the chitin hydrolysed in acid (HCl) produced oligomers with DP in the range of 2 to 6. Moreover, the oligomers with DP 4-6 were simply isolated from acetone-precipitation method.[45] In another study, the chitin oligomers produced by acetylation of chitosan oligomers with various DA up to 90% using acetic anhydride solution and isolated in HCl resulted in a value of DP ranging from 3 to 7.[42] As an alternative to acid hydrolysis, enzymatic hydrolysis has been extensively used to obtain oligomers with higher DP. As observed by Wang et *al*.[6] and Purushotham et *al*.[47], the oligomers obtained by hydrolysis with chitinases possessed DP in the range between 2 and 9. In theory, the hydrolysis must ultimately lead to the formation of chitobiose (DP 2). However, products with DP 4 and higher show better functional properties as compared to DP 2. Chitinase and lysozyme from hevamine were reported to degrade chitin polymer to form pentamers (DP 5).[70] Commercial enzymes such as hemicellulase and pectinase have also successfully depolymerized chitin to DP 6.[31] Based on the above studies, it can be concluded that both acid and enzymatic hydrolysis result in higher DP of chitin oligomers. However, the enzymatic hydrolysis condition is milder than acid hydrolysis.

Crystallinity

Chitin has a highly ordered crystalline structure, and degrading into oligomers may reduce its crystallinity. Generally, the crystallinity of chitin and its oligomer can be evaluated using X-ray diffraction measurements. The peak intensity of chitin is recorded over the scattering range of 4.5º to 50º with scan steps of 0.02º at a speed of 4.0º min^{-1}.[36,45] The crystallinity index (I_{CR}) is generally measured by a method, which uses a maximum intensity of 110 (I_{110}) and the intensity of amorphous halo contribution (I_{am}),[36,45,55,85] as follows:

$$I_{CR} = ((I_{110} - I_{am})/ I_{110}) \times 100 \quad (7)$$

The crystallinity index provides an idea on the crystalline fraction in chitin and its derivatives. The chitin exposed to adjunct treatment may be susceptible to depolymerization with crystalline fractions reduced. The crystallinity of steam explosion and depressurization treated chitin were reduced from 88 to 73%. These treated chitin samples were hydrolyzed with chitinases, and produced higher amount of oligomers compared to untreated chitin.[38] The chitin that has been treated with a combination of supercritical water and mechano-chemical grinding exhibited higher reduction of I_{CR} from 91 to 26%.[35] Ilankovan et *al*.[12] reported that chitin chemically treated with phosphoric acid, sodium hydroxide and methanol showed more amorphous nature with lower intensities of the 110 reflection. The adjunct treatment seems to result in chitin with lower crystallinity, which is more amenable to enzyme action in order to produce oligomers.

Molecular weight

Molecular weight (M) of chitin oligomers can be expressed in various ways, such as weight-average (Mw), number-average (Mn), and viscosity-average (Mv) molecular weights. Mn and Mw represent the total weight of the oligomers molecules, i.e GlcNAc and GlcN, divided by the total number of its constituting molecules and a sum of the weight fraction of each type of molecules multiplied by its molecular weight, respectively.[35] Mv can be determined from Mark-Houwink equation:

$$[\eta] = k(Mv)^{\alpha} \quad (8)$$

where [η] ($cm^3\ g^{-1}$) is the intrinsic viscosity of chitin measured by viscometer, k is 0.24 $cm^3\ g^{-1}$ and α is 0.69, where, both k and α are constant parameters of chitin.[27,86]

Measurement of the molecular weight of chitin and its oligomers commonly can be done by gel permeation chromatography (GPC) or size exclusion chromatography (SEC), which is generally equipped with refractive index detector, a GPC (gel permeation chromatography) column, and a guard column.[35,87-89] The relative mean molecular weight of the chitin is estimated by the Pullulan standard curve.[35] Prior to measurement, samples for GPC require microfiltration (with 0.45 μm filters) .[13,90]

The molecular weight is a significant parameter for effective hydrolysis of chitin. Kurita et *al*.[81] reported that the chitin molecular weight of 300 kDa was effective for enzymatic hydrolysis. It has already been mentioned that the adjunct treatments applied may reduce the molecular weight of chitin prior to hydrolysis. Dziril et *al*.[37] reported that the molecular weight of gamma-radiated chitin decreased to 60% when irradiated with 50 kGy, and it further decreased to 90% when the applied dose was 210 kGy. The application of supercritical water treatment combined with mechano-

chemical grinding reduced the molecular weight of chitin from 800 kDa to 10 kDa.[35] Ramírez-Coutiño et *al*.[39] obtained the molecular weight of 343.5 kDa for α-chitin with 90% of solubility after treating it with alkali. This molecular weight decrease is mainly due to the glycoside bond breaking caused by the treatment of chitin.[37]

On the other hand, the molecular weight of chitin oligomers produced by hydrolysis is lower than native chitin. Kazami et *al*.[45] reported that the number-average molecular weight of both α- and β-chitin rapidly decreased to 13 kDa and 10 kDa from 241 kDa and 90 kDa, respectively, after 15 min of hydrolysis in HCl. At the same time, the weight-average molecular weight of α- and β-chitin also reduced to approximately 16 kDa from 330 kDa and 250 kDa, respectively. Previous study reported that the chitin oligomers with molecular weight of 1 to 3 kDa was more effective as antioxidant agents those with molecular weight of 1 kDa and lower.[32] Thus, the biological effect of chitin oligomers is significantly dependent on the molecular weight.[91]

Oligomers yield

Yield of the oligomers after hydrolysis is a significant parameter because it determines the economic viability of the process. The yield can be expressed simply as the percentage of the chitin hydrolyzed as follows:

$$\% \text{ Yield} = \frac{W_1\text{-}W_2}{W_1} \times 100 \quad (9)$$

where, W_1 and W_2 are the initial weight and weight of chitin after hydrolysis, respectively.[31,40] The yields reported earlier for acidic hydrolysis of chitin lie between 10 and 21%.[40,45] These values are comparable with enzymatic hydrolysis. Ilankovan

et *al*.[12] and Hongkulsup[31] reported the yield of chitin oligomers hydrolyzed by commercial enzymes were in a range of 10% to 13%. Although this is a low yield, it can be increased by modifying chitin structure prior to hydrolysis by employing some of the adjunct methods discussed in this paper.

High performance liquid chromatography (HPLC) is an efficient technique for quantification of the oligomers according to each DP.[6,11,31] The amount of each DP ($GlcNAc_n$) can be estimated with the calibration curve, as shown in the following equation:

$$C_1 = C_2 \times (A_1/A_2) \quad (10)$$

where, C_1 is the sample concentration (mg/mL), C_2 is the standard concentration (mg/mL), A_1 is the peak area of sample and A_2 is the peak area of standard.[6] Wang et *al*.[6] found the yield of the $GlcNAc_2$, $GlcNAc_3$, $GlcNAc_4$ and $GlcNAc_5$ were 0.44 mg/mL, 0.08 mg/mL, 0.09 mg/mL and 0.43 mg/mL, respectively, after chitin depolymerizing under fermentation condition for two days. The results obtained show that the concentrations of $GlcNAc_2$ and $GlcNAc_5$ are significantly higher than $GlcNAc_3$ and $GlcNAc_4$. Unlike fermentation, the hydrolysis of chitin by commercial enzymes like hemicellulase and pectinase produce higher amounts of $GlcNAc_3$, $GlcNAc_4$ and $GlcNAc_5$ as reported by Hongkulsup[31]. In acid hydrolysis, higher acid concentration and temperature can influence the amount of chitin oligomers formed. The amount of $GlcNAc_2$, $GlcNAc_3$ and $GlcNAc_5$ apparently increased when the acid concentration increased from 4 N to 7 N during hydrolysis at 70 ˚C.[40] The amount of $GlcNAc_2$ produced can be 30 times greater when the hydrolysis temperature is raised to 90 ˚C.[40]

CONCLUSION

Chitin subjected to adjunct treatments allows modification of the native structure, and improve the hydrolysis rate and product functionality. Microwave irradiation on the chitin enhances access to the susceptible bonds for enzymatic hydrolysis and reduces the reaction time for hydrolysis. Crystallinity and DA of the chitin reduced when steam explosion was applied prior enzymatic hydrolysis. Furthermore, gamma irradiation, and the combination of supercritical water and grinding, can reduce chitin molecular weight and partially depolymerize it in order to facilitate enzyme attack on the substrate. It is somewhat unfortunate that the literature reviewed in this study does not specifically state the extent of yield improvement with statistical confidence, which would have enabled us to establish commercial and economic viability of using adjunct treatments. Nevertheless, one can conclude that chitin polymer reacting with acid or enzymes, particularly HCl or chitinases, can successfully produce oligomers with DP between 2 and 6. The chitin oligomers are also reported to have a potential to be commercialized for further applications, specifically as antimicrobial agents. This is due to the simple preparation method and the quality of oligomers produced without the need for deacetylation into chitosan. Unlike acid, the enzymatic hydrolysis may be considered to be more favourable due to the use of milder reaction conditions and environmental compatibility. Commercial enzymes may also be used to simplify the process and improve economics by reducing the cost of specific enzymes extraction and purification.

In conclusion, all adjunct treatments reported so far, enhance the hydrolysis of chitin, regardless of the hydrolysis method employed. For future research, it would be worth trying pre-treatments like the application of high-pressure to chitin in order to lower

its crystallinity. At the same time, it is also important to statistically quantify the improvements produced by such treatments so that their viability can be conclusively established.

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